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The apple RING-H2 protein MdCIP8 regulates anthocyanin accumulation and hypocotyl elongation by interacting with MdCOP1

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ABSTRACT

COP1, an important RING ubiquitin ligase E3, is a molecular switch for light regulation in plant development. As an interacting protein of COP1, CIP8 contains a RING-H2 domain, but its biological function is unclear. Here, the apple *MdCIP8* was identified based on its homology with *AtCIP8* in *Arabidopsis*. *MdCIP8* was constitutively expressed at different levels in various apple tissues, and the expression level of *MdCIP8* was not affected by light and dark conditions. *MdCIP8* reversed the short hypocotyl phenotype of the *cip8* mutant under light conditions. Furthermore, the yeast two-hybrid experiment showed that MdCIP8 interacted with the RING domain of MdCOP1 through its RING-H2 domain. *MdCIP8-OX/cop1-4* exhibited the phenotype of the *cop1-4* mutant, indicating that *CIP8* acts upstream of *COP1*. In addition, an apple transient injection experiment showed that MdCIP8 inhibited anthocyanin accumulation in an MdCOP1-dependent pathway. Overall, our findings reveal that CIP8 plays an inhibitory role in the light-regulation responses of plants.

1. Introduction

Zinc finger proteins contain zinc finger structures that bind one or more zinc ions and form stable, short, and self-folding "finger" polypeptides with several other specific amino acids [1]. Among them, the RING-type zinc finger protein is a large protein family, and the most typical structural feature is the zinc finger structure, which is rich in cysteine and can fix two zinc ions [2]. The RING-type zinc finger domain was first discovered from the human RING1 (Really Interesting New Gene 1) protein, which is summarized as Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys/His-X2-Cys-X(4-48)-Cys-X2-Cys (Cys is a cysteine residue, His is a histidine residue, and X is any amino acid residue) [3–5]. RING finger proteins can be divided into two groups based on whether the fifth conserved amino acid is cysteine or histidine: RING-HC (C3HC4) and RING-H2 (C3H2C3) [6,7].

Several RING finger proteins have been documented in plants. For example, bioinformatics analyses have identified 488 potential RING finger genes in rice, and 634 RING finger proteins have been identified in the apple genome [8,9]. Many RING finger proteins have ubiquitin ligase E3 activity and play an important role in the ubiquitination of proteins [10,11]. During ubiquitination, the RING domain acts as a bridge to recruit the target protein and ubiquitin-binding enzyme E2, so that E2 catalyzes the transfer of ubiquitin to the target protein, resulting in the degradation of the target protein [12]. RING finger proteins are involved in a wide variety of physiological and biochemical processes in plant cells, such as growth and development [13], light signal regulation [14], hormone signal transduction [15,16], programmed cell death [17], plant-pathogen interactions [18,19], and stress [20,21].

In plants, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) is a key negative regulator in photomorphogenesis, which is involved in many processes, such as hypocotyl elongation, anthocyanin accumulation, flowering, and circadian rhythm [22–24]. The *cop1* mutant has a shortened hypocotyl, two extended cotyledons, and increased anthocyanin accumulation and is dwarf [25]. COP1 consists of three domains: an N-terminal RING-finger motif, a middle coiled-coil domain, and C-terminal WD40 repeats. COP1 is a RING ubiquitin ligase E3, located downstream of the light signal transduction pathway, and promotes the ubiquitination degradation of ELONGATED HYPOCOTYL 5 (HY5), PRODUCTION OF ANTHOCYANINPIGMENT 1/2 (PAP1/2), CONSTANS (CO) and other light signal elements, thereby inhibiting the expression of downstream genes [26–28].

Two RING finger proteins, CSU1 and CIP8, have been reported to

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interact with COP1 in *Arabidopsis thaliana*. CSU1 is a RING ubiquitin ligase E3, which can ubiquitinate COP1 *in vitro*. CSU1 co-localizes with COP1 in the nucleus and negatively regulates the accumulation of COP1 in the dark [29]. CIP8 contains a RING-H2 domain that interacts with the RING domain of COP1 [30]. In addition, CIP8 also participates in the proteasome-mediated degradation of HY5 as an ubiquitin ligase *in vitro* [31]. However, the biological function of CIP8 remains unclear.

Apple (*Malus domestica*) is one of the most common fruit trees and is cultivated worldwide. Anthocyanins determine the color and appearance of fruits [32]. Here, we found that MdCIP8 interacted with MdCOP1 and inhibited the accumulation of anthocyanins in apple in an MdCOP1-dependent manner. Moreover, *MdCIP8* complemented the phenotype of the short hypocotyl of the *cip8* mutant under light conditions, indicating that both MdCIP8 and AtCIP8 inhibit photomorphogenesis.

2. Materials and methods

2.1. Plant materials and growth conditions

The GL3 apple shoot cultures were grown on an MS medium containing 3% sucrose, 0.5 mg/L 6-BA, 0.2 mg/L NAA and 0.1 mg/L GA3 at 25 °C under long-day conditions (16 h light/8 h dark). The 'Orin' apple calli were grown on an MS medium supplemented with 3% sucrose, 1.5 mg/L 2,4-D and 0.4 mg/L 6-BA at 25 °C in the dark [33].

For tissue expression analysis, root, stem, leave, flower and fruit were collected from 6-year-old 'Royal Gala' apple trees in the experimental station of Shandong Agricultural University. The apple fruits used for viral vectors injection were collected from mature trees of the cultivar 'Red Delicious' that were grown near Tai-An City. Fruits were bagged at 35 DAB (days after blooming); the bagged fruits were harvested at 140 DAB and de-bagged before injection.

The surface-sterilized *Arabidopsis* seeds were placed on 1/2 MS medium plates, which were kept for 3 d at 4 °C in darkness. Seedlings were grown at 22 °C under long-day conditions (16 h light/8 h dark).

2.2. Vector construction and plant transformation

To construct the expression vectors, the full-length coding sequence of *MdCIP8* were linked with pCAMBIA1300 vector. And the full-length and antisense fragment of *MdCIP8* were linked with pRI101 vector. These vectors were driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. The plasmids were transferred into *Agrobacterium tumefaciens* strain GV3101. *MdCIP8-pCAMBIA1300* was transformed into *Arabidopsis* to obtain the *35S::MdCIP8-OX* transgenic lines. *pRI101-MdCIP8* and *pRI101-asMdCIP8* was transformed into apple calli to obtain the *35S::MdCIP8-OX* and *35S::MdCIP8-anti* transgenic lines, respectively. The Pro_{35S}:: GFP-MdCIP8 vector was generated by combining the MdCIP8 entry vector (pENTR/D/TOPO) and the destination vector Pro_{35S}:: GFP-GW in an LR reaction. The transgenic lines were selected on MS medium supplemented with antibiotics [34].

2.3. Sequence alignments and phylogenetic analysis

CIP8 protein sequences of 16 plant species were obtained from the NCBI database. DNAMAN software was used to construct sequence alignment. Phylogenetic analysis was conducted with MEGA version 7.0 using the neighbor-joining method [35].

2.4. RNA extraction and quantitative RT-PCR

Total RNA was extracted from plant materials using RNA plant Plus Reagent (Tiangen) according to the instructions. The reverse transcription assay was performed using the PrimeScript first-strand cDNA synthesis kit (Takara). The UltraSYBR Mixture (CWBIO) in an ABI Step One Plus system was used to perform qRT-PCR. The specific primers are listed in Supplementary Table S1.

2.5. Yeast two-hybrid (Y2H) assay

The full-length and truncated sequences of *MdCIP8* were inserted into pGAD424. The full-length and truncated sequences of *MdCOP1* and *AtCOP1* were inserted into pGBT9. The yeast was detected on yeast dropout medium lacking Trp and Leu (-T/-L) and then lacking Trp, Leu, His and Ade (-T/-L/-H/-A). The primers for vector construction are shown in Supplementary Table S1.

2.6. Determination of the total anthocyanin content

To analyze the accumulation of anthocyanin of 15-day calli were treated on medium with low nitrogen at 17 °C under continuous white light with UV-B for one week. Anthocyanins were extracted from the samples with HCl-methanol method, and the content was calculated following the protocol described by [36].

2.7. Measurement of hypocotyl length

To measure the hypocotyl length of seedlings, *Arabidopsis* seeds sown on 1/2 MS medium were kept for 3 d at 4 °C in darkness. The seeds were then transferred to dark or light conditions (16 h light/8 h dark), and grown at 22 °C for 5 d. The hypocotyl length of seedlings was measured with ImageJ software.

2.8. Viral vector-based transient transformation in apple fruits

To construct overexpression viral vectors, the full-length sequences of *MdCIP8* were inserted into pIR vector, named pIR-MdCIP8. The IL-60-BS vector was used as a helper plasmid. Dilute the plasmid with 10 mM $MgCl_2$ solution to 10 ng/μl. The expression plasmid and the helper plasmid were mixed thoroughly at a ratio of 9:1 and then used for injection. The empty pIR vector served as a control.

To generate antisense expression viral vectors, *MdCOP1* fragments were inserted into TRV vector in the antisense orientation, TRV-MdCOP1. The TRV1 was used as an auxiliary carrier. The antisense vectors were transformed into *A. tumefaciens* strain GV3101. Dilute the bacterial pellet to OD600 = 0.6 with the infection solution (10 mM $MgCl_2$, 10 mM MES and 150 mM acetosyringone). The antisense carrier bacterial solution and the auxiliary carrier bacterial solution were mixed in a 1:1 ratio and then used for injection. The empty TRV vector served as a control.

Fruit skin infiltrations were performed as previously described [37]. The infiltrations were performed using a needleless syringe. The injected apples were kept overnight in the dark at room temperature and were then treated with 24 h continuous white light with supplemental UV-B at 17 °C for 3 to 7 d for colouration.

3. Results

3.1. Sequence analysis and phylogenetic tree of MdCIP8

To identify *CIP8* in apple, the *Arabidopsis thaliana CIP8* (*AT5G64920*) was used as bait by mining the NCBI database with the BLAST program. *MdCIP8* (*MD15G1011000*) contains an 1122-bp open reading frame that encodes 373 amino acids. Analysis of the secondary structure of MdCIP8 with the SMART tool revealed a conservative RING-H2 domain on its C-terminal side, a functional domain that is highly similar to that in *Arabidopsis* (Fig. 1A). To understand the evolutionary relationships between MdCIP8 and CIP8s from other plants, a phylogenetic tree was constructed using the amino acid sequences of CIP8s from 16 species via MEGA 7.0 software. MdCIP8 is most closely related to CIP8s from other Rosaceae plants, followed by PbCIP8, PpCIP8, and FvCIP8; MdCIP8 is most distantly related with CIP8s from monocotyledons, namely OsCIP8

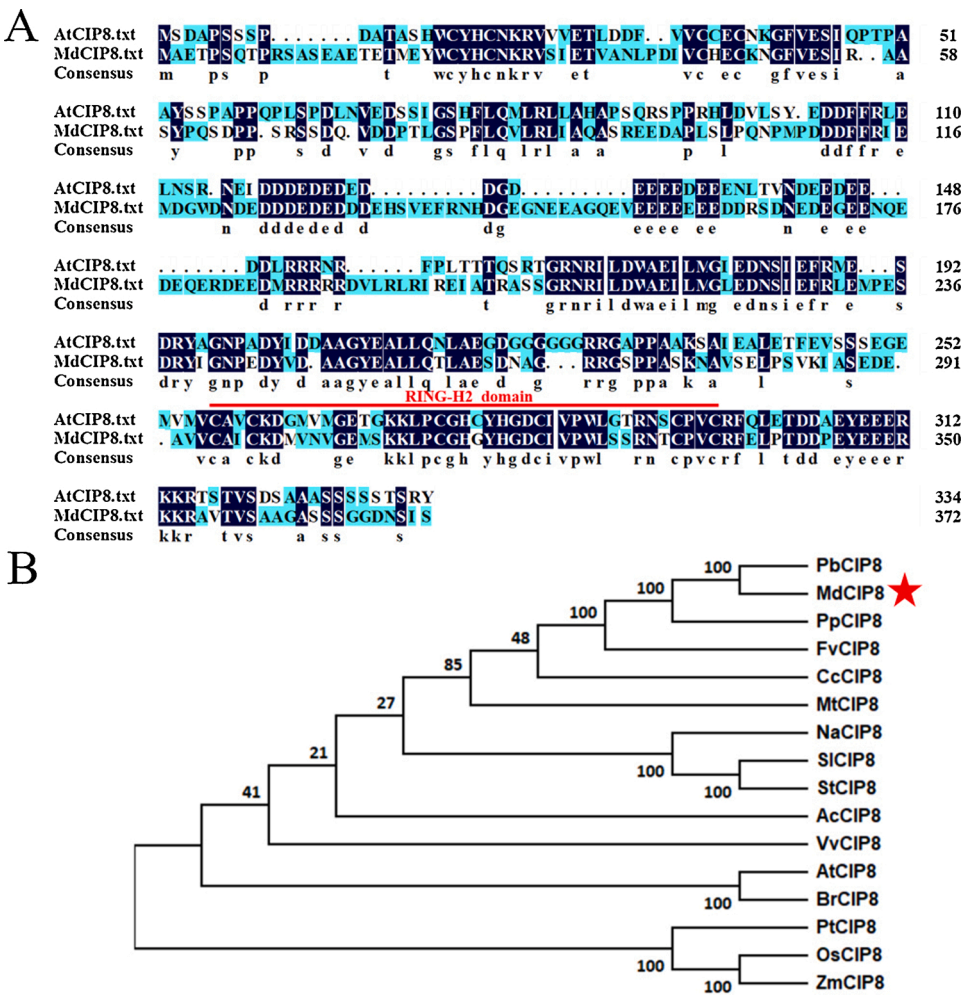

Fig. 1. Sequence analysis and phylogenetic tree of MdCIP8.
(A) Protein alignment of MdCIP8 and AtCIP8. The RING-H2 domain is marked with the red line. (B) Phylogenetic analysis of MdCIP8 and 15 other plants CIP8 protein sequences obtained from the NCBI database. MdCIP8 is denoted by the red asterisk. PbCIP8: *Pyrus bretschneideri*, MdCIP8: *Malus domestica*, PpCIP8: *Prunus persica*, FvCIP8: *Fragaria vesca*, CcCIP8: *Citrus clementina*, MtCIP8: *Medicago truncatula*, NaCIP8: *Nicotiana attenuate*, SlCIP8: *Solanum lycopersicum*, StCIP8: *Solanum tuberosum*, AcCIP8: *Ananas comosus*, VvCIP8: *Vitis vinifera*, AtCIP8: *Arabidopsis thaliana*, BrCIP8: *Brassica rapa*, PtCIP8: *Populus trichocarpa*, OsCIP8: *Oryza sativa*, ZmCIP8: *Zea mays*.

and ZmCIP8 (Fig. 1B). In addition, the protein for *MdCIP8L*, an analogous *MdCIP8* gene in apple corresponding to the gene number *MD08G1011000*, has a protein sequence similarity with MdCIP8 of 89 % (Supplemental Fig. S1). Here, our main focus is on the functional identification of MdCIP8.

3.2. Expression patterns of MdCIP8 gene

To examine tissue-specific expression patterns of *MdCIP8*, we studied the expression of *MdCIP8* in the root, stem, leaf, flower, and fruit of apple using quantitative real-time PCR (qRT-PCR). *MdCIP8* was expressed in all five organs, but the expression level was higher in leaves and lower in fruits (Fig. 2A). Light is the most important environmental factor in the process of photomorphogenesis; thus, we tested whether *MdCIP8* could respond to changes in light and dark conditions at the transcriptional level. GL3 apple culture seedlings were exposed to 0, 1, 3, 6, 12, and 24 h of light or dark treatment. The expression of *MdCIP8* did not change significantly under light and dark conditions (Fig. 2B, C), indicating that *MdCIP8* did not respond to light and dark conditions at the transcriptional level. To determine the subcellular localization of MdCIP8, the *Pro$_{35S}$*:: GFP-MdCIP8 transgenic *Arabidopsis* plant was constructed. Confocal laser scanning microscopic (CLSM) analysis of root epidermal cells from the *Pro$_{35S}$*:: GFP-MdCIP8 plant showed that MdCIP8 is located in the nucleus and cytoplasm (Fig. 2D).

3.3. MdCIP8 complements the short hypocotyl phenotype of the cip8 mutant

To explore the role of MdCIP8 in photomorphogenesis, three *MdCIP8* heterologous overexpressed *Arabidopsis* lines were selected, and the *MdCIP8-OX/cip8* complementary lines in the *cip8* mutant background were obtained by hybridization (Supplemental Fig. S2). The hypocotyl length of *Arabidopsis* was calculated after light and dark treatment. Compared with Col-0, the hypocotyl of the *cip8* mutant was shorter, and the hypocotyls of the *MdCIP8-OX/cip8#5/7/8* transgenic lines were equivalent under illumination; however, there was no significant difference in the hypocotyl length of these seedlings under dark conditions (Fig. 3A, B). Thus, MdCIP8 inhibits photomorphogenesis and complements the phenotypes of the *cip8* mutant, indicating that MdCIP8 in apple and AtCIP8 in *Arabidopsis* is functionally conservative.

3.4. MdCIP8 interacts with MdCOP1

In *Arabidopsis thaliana*, CIP8 is an interacting protein of COP1, which interacts with COP1's RING finger through its RING-H2 motif [30]. The interaction between MdCIP8 and MdCOP1 was also detected in apple. A protein domain analysis revealed that MdCIP8 has a C4 zinc finger at the N-terminal and a RING-H2 motif at the C-terminal; MdCOP1 has three domains: namely RING, coiled-coil, and WD40 (Fig. 4A, B). The entire lengths and segments of MdCIP8 and MdCOP1 were inserted into pGAD424 and pGBT9 vectors, respectively, for yeast two-hybrid (Y2H) assays. It was found that MdCIP8 interacts with MdCOP1, and their RING domains are required for their interaction (Fig. 4C), a pattern

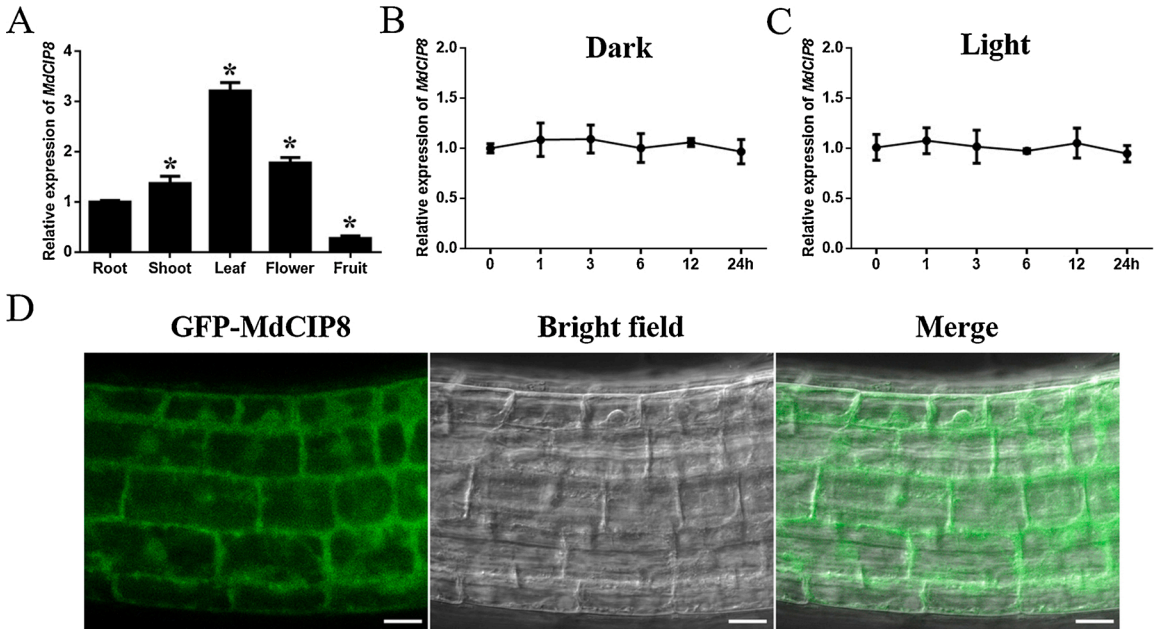

Fig. 2. Expression patterns of *MdCIP8*.
(A) *MdCIP8* transcript level in different apple tissues (root, stem, leave, flower and fruit). The value for root was set to 1. (B) Effects of dark on the transcript level of *MdCIP8*. One-month apple culture seedlings grown in light for 3 days were treated with darkness for 1, 3, 6, 12 and 24 h. The value for light-treated seedlings was set to 1. (C) Effects of light on the transcript level of *MdCIP8*. One-month apple culture seedlings grown in dark for 3 days were treated with light for 1, 3, 6, 12 and 24 h. The value for dark-treated seedlings was set to 1. Results shown are means ± SE, based on three independent biological replicates. Statistical significance was determined using Student's *t*-test: ns, $P > 0.05$; *, $P < 0.05$. (D) Subcellular localization of MdCIP8. Bars = 20 μm.

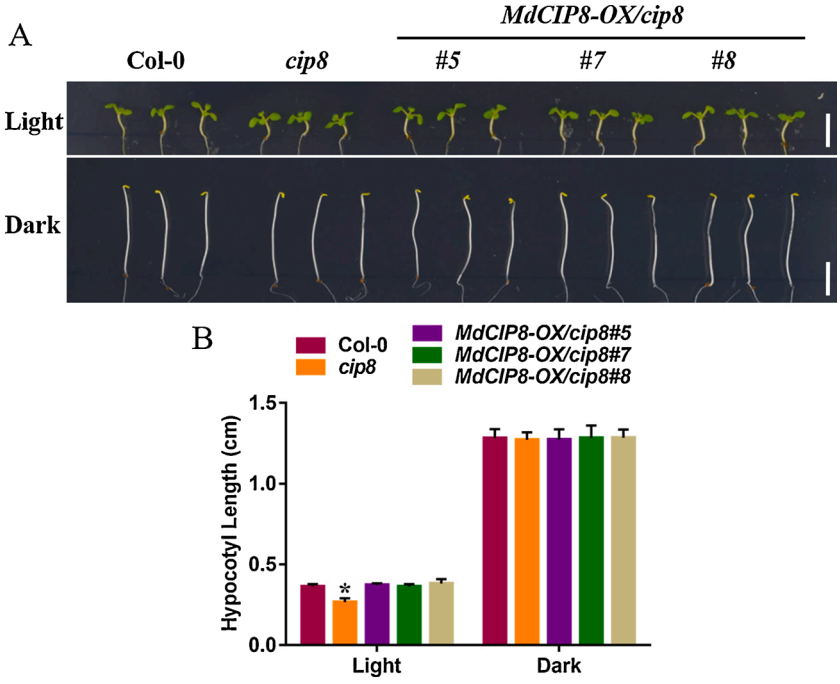

Fig. 3. *MdCIP8* complements the short hypocotyl phenotype of the *cip8* mutant.
(A) and (B) Photograph and the hypocotyl length of col-0, *cip8* and *MdCIP8-OX/cip8#5/7/8* seedlings in light and dark conditions. Col-0 was used as control. Scale bar =0.5 cm. Results shown are means ± SE, based on three independent biological replicates. Statistical significance was determined using Student's *t*-test: ns, $P > 0.05$; *, $P < 0.05$.

analogous to that in *Arabidopsis*.

3.5. CIP8 acts upstream of COP1

The functions of CIP8 and COP1 in apple and *Arabidopsis* are conservative: both inhibit photomorphogenesis and interact with each other [30,38] (Figs. 3 and 4). Then further experiments were applied to identify the possible genetic relationship between CIP8 and COP1. MdCIP8 interacts with AtCOP1 (Supplemental Fig. S3), so the stable genetic materials *Arabidopsis MdCIP8-OX*, *cop1-4*, and their hybrid material *MdCIP8-OX/cop1-4* were used. The identification results at the DNA level are shown in Supplemental Fig. S4. Under both light and dark conditions, the hypocotyls of the *cop1-4* mutant were shorter than those of Col-0. Interestingly, *MdCIP8* was overexpressed under the *cop1-4* mutant background, and the short hypocotyl phenotype of the *cop1-4* mutant was observed (Fig. 5A, B). In the adult stage, *MdCIP8-OX/cop1-4*

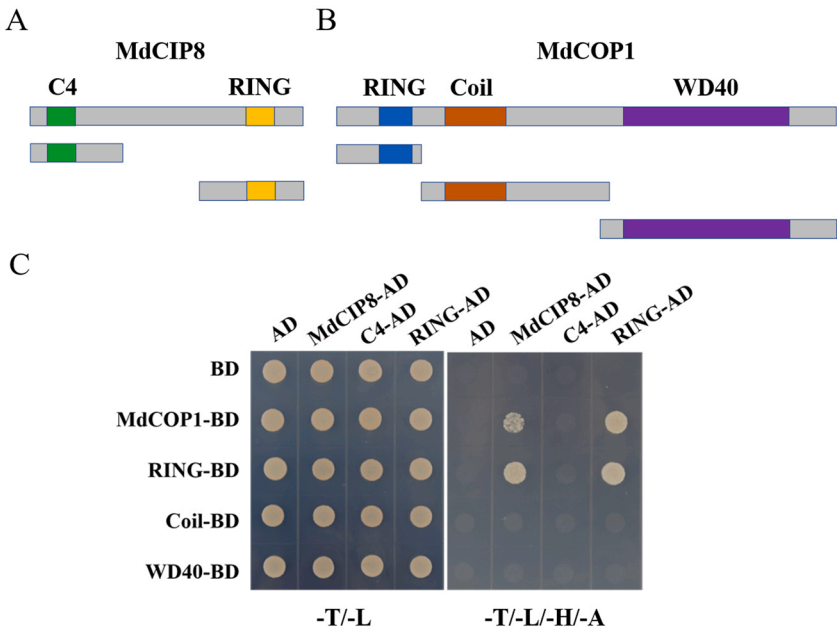

Fig. 4. MdCIP8 interacts with MdCOP1.
(A) Diagram of the different domains of MdCIP8. (B) Diagram of the different domains of MdCOP1. (C) The full-length and truncated sequences of MdCIP8 and MdCOP1 were inserted into pGAD424 and pGBT9, respectively. The RING-H2 domain of MdCIP8 specifically interacts with the RING domain of MdCOP1 in Y2H assay.

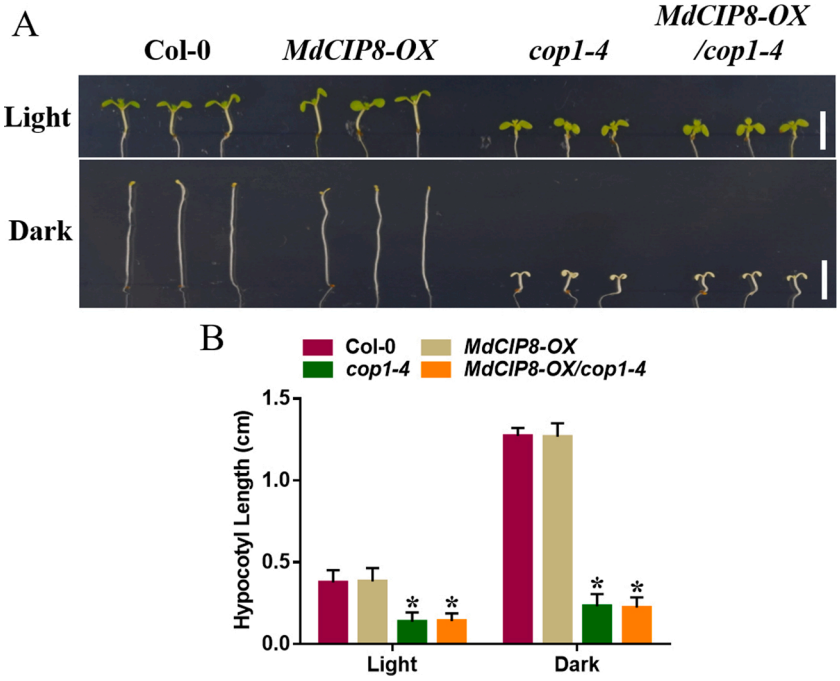

Fig. 5. *CIP8* acts upstream of *COP1*.
(A) and (B) Photograph and the hypocotyl length of col-0, *MdCIP8-OX*, *cop1-4* and *MdCIP8-OX/cop1-4* seedlings in light and dark conditions. Scale bar =0.5 cm. Results shown are means ± SE, based on three independent biological replicates. Statistical significance was determined using Student's *t*-test: ns, $P > 0.05$; *, $P < 0.05$.

also showed the typical characteristics of the *cop1-4* mutant, such as dwarfism, numerous branches, and short pods (Supplemental Fig. S5). Thus, *CIP8* acts upstream of *COP1*.

3.6. *MdCIP8 inhibits anthocyanin accumulation through MdCOP1 in apple*

In addition to the hypocotyl, anthocyanins are also a product of photomorphogenesis [39]. To further characterize the function of MdCIP8 in apple, transgenic apple calli with *MdCIP8-OX* and *MdCIP8-anti* were constructed for an anthocyanin accumulation experiment (Fig. 6B). After 7 days of continuous, strong light exposure, the MdCIP8-OX group inhibited anthocyanin accumulation, while the MdCIP8-anti group significantly promoted anthocyanin accumulation (Fig. 6A, C), indicating that MdCIP8 inhibits anthocyanin accumulation in apple calli. Anthocyanin accumulation primarily occurs in the apple fruit peel. And that a virus vector-based transient injection test was conducted to observe the effects of MdCIP8 and MdCOP1 on anthocyanin accumulation in fruits. The empty vectors pIR and TRV were used as controls. After injection of pRI-MdCIP8, the expression of *MdCIP8* was up-regulated, and anthocyanin accumulation was inhibited (Fig. 6D, E, G). After injection of TRV-MdCOP1, the expression of *MdCOP1* was down-regulated, and anthocyanin accumulation was promoted (Fig. 6D, F, G). However, when *MdCIP8* was up-regulated under the background of *MdCOP1* down-regulation, anthocyanin accumulation was promoted by TRV-MdCOP1 (Fig. 6D-G). The results suggest that MdCIP8 inhibits anthocyanin accumulation through an MdCOP1-mediated pathway in apple.

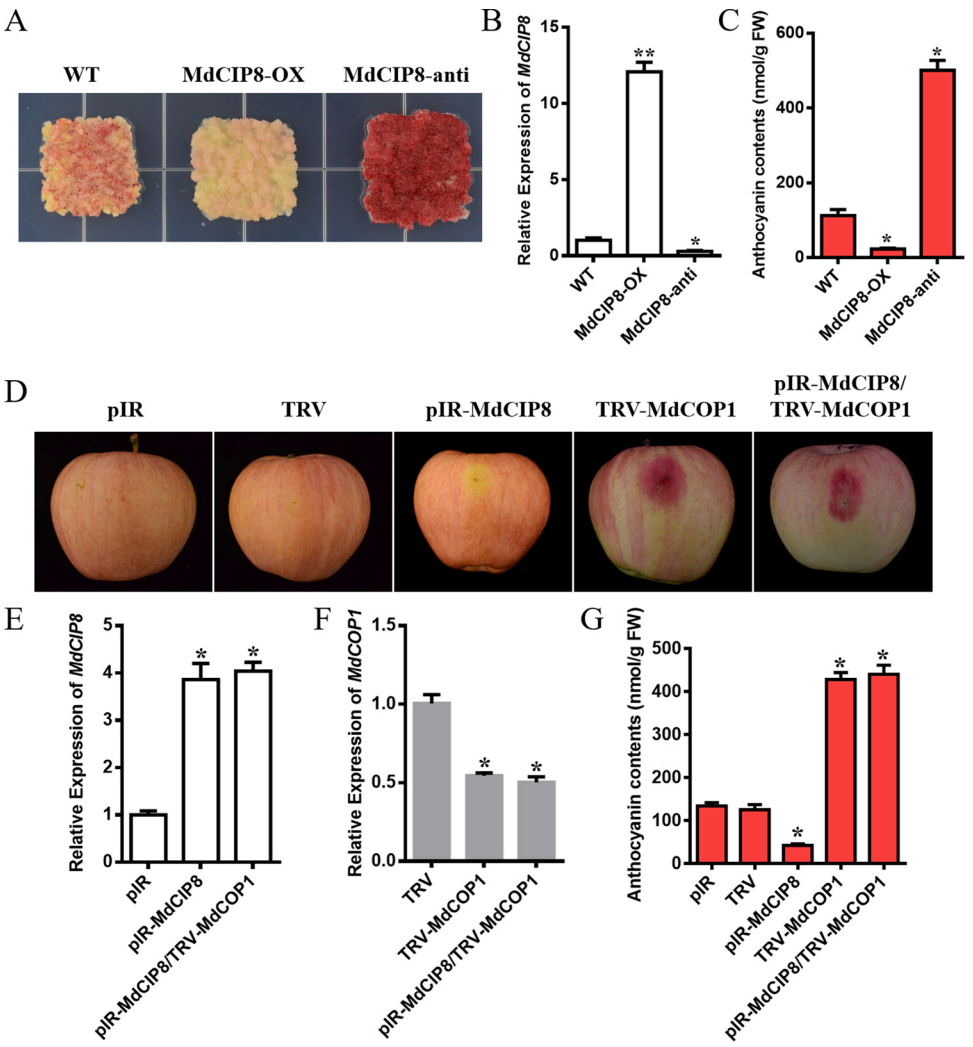

Fig. 6. MdCIP8 inhibits anthocyanin accumulation through MdCOP1 in apple.
(A) Anthocyanin phenotype of the MdCIP8-OX and MdCIP8-anti transgenic apple calli. (B) Relative expression of *MdCIP8* in the transgenic calli. The value in WT was set to 1. (C) Anthocyanin contents of the MdCIP8-OX and MdCIP8-anti transgenic calli. WT was used as control. (D) Representative images of the apple fruit peel coloration around the injection sites. Empty pIR and TRV vectors were used as controls. (E) Relative expression of *MdCIP8* after injection of pRI-MdCIP8 and pRI-MdCIP8/TRV-MdCOP1. The value in pRI was set to 1. (F) Relative expression of *MdCOP1* after injection of pRI-MdCOP1 and pRI-MdCIP8/TRV-MdCOP1. The value in TRV was set to 1. (G) Anthocyanin content of the injected fruit peel. Results shown are means ± SE, based on three independent biological replicates. Statistical significance was determined using Student's *t*-test: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$.

4. Discussion

CIP8 was first reported in *Arabidopsis*. CIP8 contains a RING-H2 domain and is an interacting protein of COP1 [30]. Here, we showed that CIP8 inhibits photomorphogenesis for the first time. The hypocotyl of the *cip8* mutant was slightly shorter under light conditions but did not change under dark conditions. However, the hypocotyls under light conditions returned to normal after overexpression of *MdCIP8* in the *cip8* mutant background (Fig. 3). This finding not only indicates that CIP8 negatively regulates photomorphogenesis but also that MdCIP8 and AtCIP8 are functionally conserved.

In both seedling and adult stages, *MdCIP8-OX/cop1-4* fully exhibited the characteristics of the *cop1-4* mutant (Fig. 5; Supplemental Fig. S5). After injection of the peel, pRI-MdCIP8/TRV-MdCOP1 promoted anthocyanin accumulation as in TRV-MdCOP1 (Fig. 6D-G). Considering the conservative functions of CIP8 and COP1 in *Arabidopsis* and apple, CIP8 is likely located genetically upstream of COP1 and relies on COP1 to perform its function in *Arabidopsis* and apple. However, the regulatory mechanisms underlying CIP8 and COP1 remain unclear.

Under dark conditions, COP1 is located in the nucleus and ubiquitinated to degrade substrates, such as HY5. Once exposed to light, COP1 is transferred from the nucleus to the cytoplasm, and the substrates are accumulated to initiate photomorphogenesis [40–42]. The RING domain may play a role in the self-association and light-responsive nucleocytoplasmic partitioning of COP1 [43,44]. CIP8 interacts with the RING domain of COP1 through its RING-H2 domain, and neither shows obvious self-association [30] (Fig. 4), implying that CIP8 may be involved in the nucleocytoplasmic shuttling of COP1. *CIP8* does not respond to light and dark conditions at the transcriptional level, but it does at the protein level [30] (Fig. 2B, C). In the wild type, the protein content of CIP8 in light-grown seedlings was higher than that in dark-grown seedlings. However, the protein content of CIP8 was reduced in severe *cop1* mutants, indicating that the stability of CIP8 depends on COP1 [30,45]. Thus, CIP8 and COP1 may influence each other; however, the regulatory mechanisms underlying their interaction require further study.

Anthocyanin is an important secondary metabolite of plants, which not only gives fruits rich color and attracts consumers, but also has very important nutritional and pharmacological effects [46–48]. Anthocyanin biosynthesis is catalyzed by a series of enzymes encoded by structural genes and regulated by many transcription factors [49,50]. Light induced MdMYB1 expression on apple peel, and MdMYB1 activated the transcription of structural genes *MdCHS*, *MdF3H*, *MdDFR* and *MdUFGT* in the anthocyanin synthesis pathway, promoting anthocyanin accumulation [51,52]. After shading treatment, MdCOP1 was transferred to the nucleus, and ubiquitination degrades MdMYB1, resulting in decreased anthocyanin content and lighter peel color [38]. This regulatory mechanism was subsequently confirmed in *Arabidopsis* [28,53]. HY5, another key target protein of COP1, can bind to the promoter of *CHS* and activate its expression, thus promoting anthocyanin biosynthesis [26,54,55]. This study revealed that MdCIP8, an MdCOP1 interacting protein, inhibits anthocyanin accumulation in an

MdCOP1-dependent manner (Fig. 6). This not only enriched the molecular network of COP1 regulating anthocyanin, but also provided a theoretical reference for improving fruit appearance quality.

Author contributions

Yu-Jin Hao, Xiao-Fei Wang, and Hui Kang conceived and designed the research. Hui Kang, Ting-Ting Zhang, and Lu-Lu Fu preformed the experiments. Chun-Xiang You provided technical assistance. Hui Kang, Xiao-Fei Wang and Yu-Jin Hao analysed the data and wrote the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110665.

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